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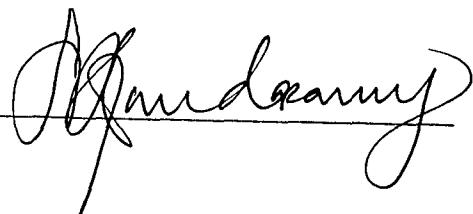
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We have developed methods for the identification and characterization of SR and SR-associated proteins with the aim of developing a more detailed understanding of interactions that are important for the regulation of pre-mRNA splicing. A splicing complex affinity-selection assay has been established that allows the sensitive detection of SR protein interactions at different stages of splicing complex formation. Using this assay, we have investigated the mechanism by which exonic splicing enhancer sequences (ESEs) function. In particular, we have tested a previous model in which SR proteins bound to an ESE promote the formation of splicing complexes at adjacent splice sites by stimulating the binding of the U2 snRNP auxiliary factor (U2AF) to the polypyrimidine tract. The results clearly demonstrate that the recruitment of SR proteins by an ESE to pre-mRNA does not influence the binding of the U2AF-65kDa subunit to the pre-mRNA. Moreover, evidence is obtained using the affinity selection assay that, instead of the ESE, the presence of U1 snRNP is critical for U2AF binding.				
Using biochemical fractionation in combination with immunoblotting with anti-SR protein antibodies, we have obtained fractions that are highly enriched for several SR proteins and candidate SR-associated factors. Mass spectroscopy identification of the fractionated polypeptides has identified the myeloid leukemia and A-T associated protein DEK as a co-fractionating component. Consistent with an interaction between DEK and splicing components, DEK is co-immunoprecipitated with antibodies to the SRm160/300 splicing coactivator. Moreover, an antibody that is highly specific for DEK immunoprecipitates splicing complexes. The results demonstrate that DEK, which is implicated in different cancers, is an SR-associated protein that assembles in splicing complexes. The functional significance of these interactions is currently being investigated. The results may provide new insights into mechanisms by which splicing is deregulated in certain cancers.				
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Introduction

Deregulation of alternative splicing has been linked to malignant transformation and the formation of metastases in breast cancers. For example, specific alternatively spliced forms of the pre-mRNA encoding the cell surface adhesion glycoprotein CD44 have been correlated with invasive tumor formation. Moreover, it has been demonstrated that expression of specific alternative spliced forms of CD44 mRNAs in non-metastatic cell lines result in the transition to full metastatic potential. We are interested in understanding the mechanisms underlying the regulation of alternative splicing with the goal of identifying and targeting trans-acting splicing factors that are involved in the formation of breast and other types of cancer.

Pre-mRNA splicing requires the assembly of a large multi-subunit complex, the spliceosome, which consists of four small nuclear ribonucleoprotein particles (U1, U2, U4/U6 and U5 snRNPs) and numerous non-snRNP protein factors (Kramer, 1996; Burge et al., 1999). Biochemical and genetic studies have provided evidence that over 50 proteins are required for constitutive splicing, many of which are highly conserved between yeast and mammals. In metazoans, a large number of additional non-snRNP splicing factors have been identified which contain domains rich in alternating serine and arginine residues (RS domains) (Fu, 1995; Manley and Tacke, 1996; Valcarcel and Green, 1996). A subgroup of these proteins, the SR family, contain one or two N-terminal RNA recognition motifs and a phosphorylated C-terminal RS domain. SR family proteins include SRp20,

ASF/SF2, SC35, 9G8, SRp30c, SRp40/HRS, SRp55, SRp75, and a more distantly related protein, p54. These proteins promote general splicing activity and also function in regulated pre-mRNA splicing. SR proteins interact with each other and also with snRNP-associated proteins that contain RS domains and these interactions are mediated by phosphorylated RS domains [Wu, 1993 #263; Kohtz, 1994 #152; Fetzer, 1997 #38; Amrein, 1994 #199; Xiao, 1997 #192; Xiao, 1998 #607; Yeakley, 1999 #616]. These protein-protein interactions are important for promoting the recognition and pairing of splice sites and for subsequent steps in spliceosome assembly.

The assembly of spliceosomes involves an ordered association of snRNPs and non-snRNP protein factors with the pre-mRNA (Kramer, 1996). At an early stage in assembly, U1 snRNP binds to sequences at the 5' splice site and promotes the binding of U2 snRNP to the intron branch site (Seraphin and Rosbash, 1989; Barabino et al., 1990). The stable binding of U2 snRNP to the branch site also requires the U2 auxiliary factor (U2AF), which consists of two subunits, U2AF-65kDa and U2AF-35kDa (Ruskin et al., 1988). U2AF-65kDa contains a short N-terminal RS domain followed by three RRMs and U2AF-35kDa contains a single RRM and a short C-terminal RS domain (Zamore et al., 1992; Zhang et al., 1992). U2AF binds through the 65kDa subunit to the polypyrimidine tract located between the branch site and 3' splice site (Zamore and Green, 1991). Following the assembly of a U2 snRNP-containing pre-splicing complex, U4/U6 and U5

snRNPs enter the assembly pathway as a pre-formed tri-snRNP particle which, together with additional protein factors, form an assembled spliceosome.

In higher eukaryotes, the sequences specifying splice sites and the branch site are weakly conserved and are often surrounded by multiple cryptic splice sites that are not selected. Moreover, many pre-mRNAs in metazoan cells contain alternative splice sites that must be recognized in a regulated fashion to generate specific mRNA variants (reviewed in (Wang and Manley, 1997; Lopez, 1998)). Additional sequence elements within a pre-mRNA are important for splice site recognition and one type of element is the exonic splicing enhancer (ESE). One or more SR proteins bind to ESEs and promote the recognition of adjacent splice sites (reviewed in (Fu, 1995; Chabot, 1996; Manley and Tacke, 1996; Valcarcel and Green, 1996)). ESEs are often purine-rich although recent studies employing SELEX procedures have demonstrated that diverse sequences can function as enhancers in conjunction with different SR proteins (Liu et al., 1998; Schaal and Maniatis, 1999b). Recently it was demonstrated that the splicing coactivator SRm160/300 (complex of SR-related matrix proteins of 160kDa and 300kDa) is required for ESE-dependent splicing (Blencowe et al., 1998; Eldridge et al., 1999). The association of SRm160/300 with an ESE-dependent pre-mRNA requires U1 snRNP, factors bound to the ESE, and is stabilized by U2 snRNP. Independently of pre-mRNA, SRm160/300 interacts specifically with U2 snRNP and hTra2 β , a human homolog of the *Drosophila* alternative splicing regulator Transformer -2 that binds to purine-rich ESEs (Tacke et al., 1998). It was proposed that SRm160/300 functions in ESE-dependent splicing

by mediating critical interactions between ESE-bound components and the snRNP machinery of the spliceosome.

In previous studies it was proposed that one or more SR proteins bound to an ESE promote splicing by stabilizing the binding of U2AF-65kDa to the polypyrimidine tract through protein-protein interactions mediated by U2AF-35kDa (Wu and Maniatis, 1993; Wang et al., 1995; Zuo and Maniatis, 1996). In research performed during the first year of the Idea Award, we have tested this model and find that, contrary to previous observations, a purine-rich ESE does not facilitate the binding of U2AF-65kDa to the polypyrimidine tract. Part I of this report presents the results of this study and other evidence that ESEs function by a set of interactions that are distinct from those required for the binding of U2AF-65kDa to the polypyrimidine tract.

Part II of this report describes the development of a strategy for the isolation of new SR-related and SR-associated proteins that function in splicing. This strategy has lead to identification of the putative oncoprotein DEK as an SR-associated protein that assembles into splicing complexes. The possible functional significance of this association and its disease relevance is discussed.

(Part I: Body)

Part I. Distinct factor requirements for exonic splicing enhancer function and binding of the U2 snRNP auxiliary factor to the polypyrimidine tract

ESE-dependent association of SR family proteins and hTra2 β with a dsx-pre-mRNA

We have investigated the protein composition of splicing complexes assembled on an ESE-dependent substrate derived from exons 3 and 4 of the *Drosophila doublesex* (dsx) gene, which contains a suboptimal polypyrimidine tract and a typical mammalian ESE consisting of GAA repeats in the 3' exon (Yeakley et al., 1996). This pre-mRNA is spliced with increased efficiency as the number of GAA repeats in the ESE is increased (Yeakley et al., 1996; Eldridge et al., 1999). Biotinylated derivatives of the dsx pre-mRNA were synthesized that lack an ESE (dsx Δ E+bio), that contain an ESE with either three (dsx(GAA)₃+bio) or six (dsx(GAA)₆+bio) GAA-repeats. These biotinylated pre-mRNAs are spliced with similar efficiency as the unmodified substrates (Fig. 1A and data not shown). Splicing complexes assembled on the biotinylated pre-mRNAs were affinity-selected on streptavidin agarose beads and analyzed by immunoblotting with mAb104, which detects a phosphoepitope within the RS domains of many SR proteins (Fig. 1B).

The GAA-repeat ESE promoted the association of several SR-related proteins with the *dsx* pre-mRNA, including species migrating at 30kDa, 40kDa, 55kDa and 75kDa. Low levels of these proteins were detected in complexes on the *dsx* Δ E pre-mRNA (lane 5). These levels increased significantly on the *dsx*(GAA)₃ pre-mRNA (lane 6). A further increase was detected for the 40kDa, 55kDa and 75kDa proteins on the *dsx*(GAA)₆ pre-mRNA (lane 7). Of the different proteins detected by mAb104, the 40kDa species was the most strongly enriched in response to the increase in number of GAA-repeats. The amount of each mAb104-reactive protein that associates with the *dsx*(GAA)₆ pre-mRNA, depending on the species, is approximately 1-3% of the total amount of the protein in the splicing reaction. The selection of the different SR proteins on the *dsx* pre-mRNAs was not due to non-specific interactions with the streptavidin agarose beads since none of the proteins were detected in selections from reactions incubated without pre-mRNA (lane 3), or with a *dsx*(GAA)₆ pre-mRNA lacking biotin residues (lane 4). Moreover, only a specific subset of SR-related proteins was selected since at least one prominent protein detected by mAb104 in total extract, migrating at ~130kDa, was not enriched on the *dsx* (GAA)₆ pre-mRNA (compare lanes 1 and 7).

Complexes affinity selected on the different *dsx* pre-mRNAs were also immunoblotted with antibodies specific for SRm160 and SRm300. Previous immunoprecipitation experiments performed under low salt (100mM NaCl) conditions demonstrated that increased levels of these proteins associate with the

dsx pre-mRNA as the number of GAA-repeats in the ESE increase (Eldridge et al., 1999). However, it was found that they dissociate under the higher salt conditions (300mM KCl) used in the affinity selections assays in the present study (data not shown). This indicates that SRm160/300 and possibly additional SR-related proteins are more weakly associated with the dsx pre-mRNA than hTra2 β and the SR proteins detected by mAb104.

The 30kDa, 55kDa and 75kDa proteins detected by mAb104 most likely correspond to the defined SR family proteins of these sizes. Candidates for the 40kDa species include the SR family protein SRp40/HRS (Diamond et al., 1993; Scream et al., 1995) and hTra2 α and or hTra2 β . The latter two proteins are human homologs of the *Drosophila* alternative splicing factor Transformer-2 (Dauwalder et al., 1996; Beil et al., 1997), which are detected by mAb104 and bind specifically to ESEs containing GAA-repeats (Tacke et al., 1998). Consistent with the 40kDa species containing one of the hTra2 proteins, a polyclonal antibody specific for hTra2 β (Tacke et al., 1998) detected increased levels of this protein in the dsx splicing complexes as the number of GAA repeats increase (Fig. 1C). The level of enrichment of hTra2 β was similar to that observed for the 40kDa species detected by mAb104, indicating that a significant fraction of the latter may correspond to hTra2 β (compare Figs. 1B and 1C). The results in Fig. 1 provide evidence that a GAA-repeat ESE promotes the association of a specific set of SR proteins and hTra2 β with the dsx pre-mRNA.

Binding of U2AF-65kDa to the dsx pre-mRNA does not require an ESE

In previous studies, it was proposed that ESEs function by promoting the binding of U2AF-65kDa to adjacent, suboptimal, polypyrimidine tract (Zuo and Manley, 1993; Wang et al., 1995). To determine whether this is the case for the dsx pre-mRNA in the present study, protein samples from the affinity selections in Fig. 1B were probed with a polyclonal antibody specific for U2AF-65kDa (Fig. 2A). In contrast to the ESE-dependent association of SR proteins and hTra2 β with the dsx pre-mRNA, U2AF-65kDa bound to this substrate in the absence of the ESE (lane 3). Moreover, its level of binding did not change significantly in the presence of the ESE (compare lanes 3 and 5). The slight increase in the level of U2AF-65kDa detected in the selection with the dsx(GAA)₃ pre-mRNA, compared to the levels observed in the selections with the dsx Δ E and dsx(GAA)₆ pre-mRNAs (compare lane 4 with lanes 3 and 5) was not observed in several repeat experiments and was attributed to a minor loading difference. As in the case of the SR proteins detected in the dsx splicing complexes, binding of U2AF-65kDa to the dsx pre-mRNA was not due to a non-specific interaction since it was not selected from reactions containing the non-biotinylated dsx(GAA)₆ pre-mRNA (lane 2), nor was it selected from a reaction containing a biotinylated 5' half pre-mRNA lacking a polypyrimidine tract (Fig. 2B, compare lanes 2 and 3). The results indicate that binding of U2AF-65kDa to the dsx pre-mRNA is not dependent on the ESE.

Kinetics of association of SR proteins and U2AF-65kDa with the dsx pre-mRNA

To extend the above observations, we next analyzed the kinetics of association of SR proteins and U2AF-65kDa with the dsx (GAA)₆+bio pre-mRNA (Fig. 3). Complexes assembled on this substrate were affinity selected after different incubation times in a splicing reaction. Proteins recovered from the splicing complexes were immunoblotted with mAb104 (Fig. 3A) and the anti-U2AF-65kDa antibody (Fig. 3C). Interestingly, different SR proteins were detected by mAb104 on the pre-mRNA at different times during the reaction. “SRp75” bound on ice, and its level did not change significantly during incubation of the splicing reaction at 30°C (compare lanes 3-5). By contrast, the other SR proteins and hTra2β were detected at very low levels on ice and increased rapidly during the incubation at 30°C, reaching essentially maximal levels during the first ten minutes of the reaction (compare lanes 3-5; data not shown). Consistent with an energy requirement for the association of these SR proteins with the pre-mRNA, they bound at significantly lower levels in splicing reactions incubated without ATP (data not shown). The levels of the SR proteins selected at 10 min in the presence of ATP (lane 5) remained constant for a further ~30 min, after which they declined (data not shown). As observed in Fig. 1B, the 40kDa species detected by mAb104, corresponding in part to hTra2β, was enriched to a higher extent than the other SR proteins on the dsx(GAA)₆ pre-mRNA. An analysis of the supernatant fractions from the selections by immunoblotting with mAb104 did

not reveal significant differences in the levels of the non-selected proteins, which comprise at least 97% of the total amounts of these proteins in the splicing reaction (Figure 3B, compare lanes 1-3). This indicates that the increased levels of detection of the SR proteins on the *dsx*(GAA)₆ pre-mRNA is not due to changes in the levels and/or phosphoepitope status of the SR proteins prior to their association with the pre-mRNA. However, a slower migrating 30kDa species was enriched in splicing complexes selected at 10 min compared to complexes selected at 0 and 5 min (Fig. 3A, compare lanes 1-3). It is possible that this protein represents a distinct 30kDa species, or else a modification to an existing protein within the affinity selected splicing complexes (see discussion below). The results in Fig. 3A indicate that the ESE-dependent recruitment of several SR family proteins and hTra2 β on the *dsx*(GAA)₆ pre-mRNA occurs during the first 10 min of a splicing reaction. Moreover, these proteins differ in the levels and kinetics with which they associate with the *dsx* pre-mRNA.

The complexes affinity-selected in Fig. 3A were next immunoblotted with the anti-U2AF-65kDa antibody (Fig. 3C). U2AF-65kDa bound to the *dsx*(GAA)₆ pre-mRNA on ice and, in contrast to the increase in binding of the SR proteins and hTra2 β , its level of binding did not change significantly during the splicing reaction (compare lanes 3-5). These results indicate that binding of U2AF-65kDa to the *dsx*(GAA)₆ pre-mRNA is rapid and occurs prior to the stable association of several SR family proteins and hTra2 β .

Binding of U2AF-65kDa to the dsx pre-mRNA requires U1 snRNP

We next investigated the factor requirements for binding of U2AF-65kDa to the dsx substrate. It has been reported recently that U1 snRNP is required for the binding of U2 snRNP to the dsx(GAA)₆ pre-mRNA used in the present study (Eldridge et al., 1999). It has also been reported that U1 snRNP can promote the cross-linking of U2AF-65kDa to an upstream polypyrimidine tract across an exon and also to the polypyrimidine tract of a constitutively spliced pre-mRNA containing a single intron (Hoffman and Grabowski, 1992; Cote et al., 1995). We therefore investigated if it is also required for the binding of U2AF-65kDa within the context of cross-intron interactions during ESE-dependent splicing on the biotinylated dsx(GAA)₆ pre-mRNA. Splicing complexes assembled on this substrate were affinity selected from splicing reactions depleted of individual snRNPs and then immunoblotted with the anti-U2AF-65kDa antibody (Fig. 4). Depletion of U1 snRNP resulted in a significant reduction in the level of U2AF-65kDa binding to the dsx(GAA)₆ pre-mRNA compared to its level of binding in a “mock”-depleted extract (compare lanes 2 and 4). This reduction was not due to a non-specific loss since depletion of U2 snRNP did not reduce the level of U2AF-65kDa binding (compare lanes 2 and 5), and mixing equal amounts of the U1 and U2 snRNP-depleted extracts restored binding to the level observed in the mock-depleted reaction (compare lanes 2 and 6). These results demonstrate that U1 snRNP functions in stabilizing the binding of U2AF-65kDa to the dsx(GAA)₆ pre-mRNA.

Discussion and Conclusions (Part 1)

The results in present study provide evidence that a purine-rich ESE is important for the stable association of hTra2 β and several SR family proteins with the dsx pre-mRNA, but not U2AF-65kDa. Instead, binding of U2AF-65kDa is promoted by U1 snRNP. This indicates that an ESE promotes the formation of splicing complexes through interactions that are separate from those required for the binding of U2AF-65kDa to the polypyrimidine tract. These findings are in contrast to those of previous studies in which it was reported that cross-linking of U2AF 65kDa to pre-mRNAs containing suboptimal polypyrimidine tracts is increased in the presence of an ESE (Lavigne et al., 1993; Wang et al., 1995; Bouck et al., 1998). However, in these studies the level of U2AF-65kDa cross-linking was not found to correlate with increased splicing activity promoted by the ESE. In another study employing recombinant factors in the absence of nuclear extract, it was observed that efficient cross-linking of U2AF-65kDa to the dsx pre-mRNA required the addition of a single SR family protein and U2AF-35kDa (Zuo and Maniatis, 1996). In the present study, binding of U2AF-65kDa to the dsx pre-mRNAs was assayed in splicing reactions containing total nuclear extract without added factors. It is possible that the experimentally defined levels of SR proteins and U2AF subunits in the previous study resulted in the promotion of interactions that do not normally occur in splicing reactions. The present findings are consistent with a very recent report demonstrating that crosslinking

of U2AF-65kDa to two different ESE-dependent pre-mRNAs occurs with equal efficiency in the presence or absence of the ESE, and that immunodepletion of U2AF-35kDa does not prevent ESE-dependent splicing in vitro (Kan and Green, 1999). Moreover, it has also been reported recently that the RS domain of the *Drosophila* homolog of U2AF-35kDa, which was proposed to mediate interactions with ESE-bound SR proteins (Wu and Maniatis, 1993), is dispensable for viability and also the regulation of *dsx* pre-mRNA splicing (Rudner et al., 1998).

An ESE consisting of six GAA repeats was sufficient for promoting the association of several SR proteins and hTra2 β with the *dsx* pre-mRNA. This is consistent with previous studies demonstrating that SR proteins of these sizes can associate specifically with short RNAs consisting only of GAA-repeats (Yeakley et al., 1996; Schaal and Maniatis, 1999b). Interestingly, in the present study these proteins were detected on the *dsx*(GAA)₆ pre-mRNA at different times during the splicing reaction, with the stable binding of “SRp75” appearing earlier than the 55kDa, 40kDa/hTra2b and 30kDa SR protein(s). This indicates that different SR proteins and hTra2 β may associate with the *dsx* pre-mRNA individually rather than as a pre-assembled complex. The increased detection of a slower migrating 30kDa SR protein(s) on the *dsx*(GAA)₆ pre-mRNA could reflect a modification such as phosphorylation to one or more proteins of this size in the selected splicing complexes and may relate to a previous observation that the phosphorylation of ASF/SF2 is important for ESE-dependent splicing (Xiao and

Manley, 1998). However, it remains to be determined if this slower migrating species represents a modified protein and or a distinct 30kDa species recruited during the splicing reaction. In any case, the results in the present study demonstrate that the SR protein composition of complexes formed on the *dsx* pre-mRNA undergoes dynamic changes during ESE-dependent splicing.

We have demonstrated recently that the level of binding of U1 snRNP to the *dsx* pre-mRNA substrate in the present study is approximately equivalent in the presence and absence of the ESE (Eldridge et al., 1999). It was therefore unexpected that only minor levels of SR family proteins and hTra2 β were detected on the *dsx* pre-mRNA in the absence of the ESE, since previous reports have indicated that SR proteins such as ASF/SF2 promote the binding to U1 snRNP to the 5' splice site (Kohtz et al., 1994; Xiao and Manley, 1998). However, as in the previous U2AF binding studies cited above, these earlier studies assayed binding of U1 snRNP in reactions containing added purified components, whereas we have assayed binding in splicing reactions without added purified proteins. Although the low levels of SR proteins detected on the *dsx* Δ E pre-mRNA could represent a population that functions in promoting the binding of U1 snRNP, the present results, taken together with those of Eldridge et al. (1999) (Eldridge et al., 1999), indicate that the increased SR protein and hTra2 β association with the *dsx* pre-mRNA promoted by the ESE does not significantly influence U1 snRNP binding.

We also demonstrated recently that U1 snRNP is strongly required for the association of both U2 snRNP and the SRm160/300 splicing coactivator with the dsx(GAA)₆ pre-mRNA (Eldridge et al., 1999). By contrast, in the presence of U1 snRNP, but absence of the ESE, these components bound weakly to this substrate. Taken together with the results of the present study, we propose that U1 snRNP promotes two distinct sets of interactions during ESE-dependent splicing. One set involves ESE-independent interactions that are required for the binding of U2AF-65kDa to the polypyrimidine tract, which then promotes partial binding of U2 snRNP to the branch site. This set of interactions likely involves previously proposed cross-intron interactions mediated by the branch site-binding factor SF1/mBBP, which interacts with U2AF-65kDa and is also required for the stable binding of U2 snRNP to the branch site (Kramer, 1992; Abovich and Rosbash, 1997). The other set of interactions promoted by U1 snRNP simultaneously requires the ESE and functions to further stabilize the binding of U2 snRNP to the branch site, and also promotes the association of SRm160/300 with the pre-mRNA (Eldridge et al., 1999). This set of interactions does not influence the binding of U2AF to the pre-mRNA. Although depletion of SRm160/300 or U2 snRNP weakens but does not prevent the association of the other component with the dsx(GAA)₆ pre-mRNA, these two components interact specifically (Eldridge et al., 1999). Thus, instead of promoting splicing complex formation through interactions mediated by the U2AF heterodimer, one or more ESE-associated components, including the SRm160/300 splicing coactivator, may promote splicing by interacting directly with the snRNP machinery of the spliceosome.

Part II (Body)

The putative oncoprotein DEK associates with SR proteins and pre-mRNA splicing complexes.

A strategy for the isolation of new SR-related and SR-associated proteins.

In order to identify new SR-related and SR-associated proteins that function in RNA processing, we have used column chromatography to fractionate proteins that react with the anti-SR protein antibodies mAb-104, NM4 and NM22. All three of these antibodies recognize distinct phosphoepitopes that are shared between proteins containing RS domains. Using mAb104 to initially monitor purification, we fractionated HeLa nuclear extract by the scheme shown in Figure 5A. The final (Q-Sepharose) column yielded subfractions that were highly enriched for a doublet mAb-104 antigens that migrate at 130kDa, as well as antigens corresponding in size to members of the SR family of splicing factors, including 75, 50 and 33kDa proteins. This indicates that these SR proteins share similar chromatographic properties or else are associated with one another in a complex. The presence of the 130kDa doublet detected by coomassie-staining in Q-Seph-0.3 correlated precisely with the detection of the 130kDa antigen, indicating that this band is highly enriched in this antigen. Figure 5B shows an immunoblot of the final fraction (Q-Seph-0.3) from the purification scheme

probed with mAb104, and Figure 5C shows a corresponding coomassie-stained gel of the same fraction. Besides the 130kDa antigen, additional antigens of 120, 50, 35, 20 and 17 kDa were detected in the fraction by coomassie staining (indicated by asterisks in Figure 5C; see below).

To determine the identity of the 130kDa mAb-104 antigen and the other abundant components in the Q-Seph-0.3 fraction, the prominent bands in the gel in Figure 5C were excised, digested with trypsin *in situ*, and the released peptides were analyzed by MALDI-TOF mass spectroscopy. The masses of peptides from each protein were used to search corresponding peptide masses in conceptually translated sequences from the databases. Exact matches were obtained between peptides from both proteins in the p130 doublet and the 120, 50, 35, 20 kDa proteins, identifying these with 100% confidence as Hel117, ABC50, DEK, SC35 and eIF1A, respectively (data not shown). Smaller SR proteins besides SC35 that were detected by immunoblotting with mAb104 were not detected in the coomassie stained gel most likely because they are lower in abundance. Consistent with the immunological relationship between p130 and SR proteins, Hel117 is a previously reported protein of unknown function that contains an RS domain (Sukegawa and Blobel, 1995). It contains other domain features found in splicing factors including a DEAD-box and associated motifs shared between proteins that have dsRNA unwinding/ nucleic acid-dependent ATPase activity. Consistent with a relationship with splicing, Hel117 was previously localized to nuclear speckle structures enriched in pre-mRNA splicing components (Sukegawa

and Blobel, 1995). ABC50 is a previously reported protein of unknown function that contains consensus motifs corresponding to nucleotide binding folds in the ATP-binding cassette (ABC) class of proteins, many of which are involved in the transport of molecules across biological membranes (Richard et al., 1998). However, in contrast to most other ABC proteins, ABC lacks transmembrane domains. Interestingly it contains several putative nuclear localization signals as well as regions of homology with proteins involved in translation (Richard et al., 1998). DEK is a multi-disease-associated protein that was originally identified as an in-frame fusion partner with the nucleoporin CAN, generated by a specific (6;9) chromosomal translocation in a subset of acute myeloid leukemias (AML) (von Lindern et al., 1992). The normal function of DEK and its role in the formation of AML is not known. DEK has also been identified as an autoimmune antigen in patients with early onset rheumatoid arthritis. eIF1A is a factor required for the initiation of translation. The co-purification of Hel117, ABC, DEK and eIF1A with SR proteins, including SC35, suggests that these factors either have similar chromatographic properties to SR proteins, or else that they are physically associated with one or more SR proteins in a complex(es). Using an available antibody that is highly specific for DEK (rAb-DEK; Fornerod et al., 1995), we next determined whether it is associated with SR proteins.

DEK is associated with the SRm160/300 splicing coactivator

Antibodies (mAb-B1C8 and rAb-SRm160) that are highly specific for the splicing coactivator subunit SRm160, in addition to SRm160/300, coimmunoprecipitate several SR family proteins detected by mAb104, as well as hTra2 β (Eldridge et al., 1999) (unpublished observations). To determine whether DEK is also coimmunoprecipitated with SRm160, mAb-B1C8 immunoprecipitates were prepared from HeLa cell nuclear extract pre-incubated with or without RNase. MAb-B1C8, but not a monoclonal antibody of the same isotype that is specific for the hyperphosphorylated RNA polymerase II large subunit (mAb-B3), specifically co-immunoprecipitated DEK (Figure 6). This co-immunoprecipitation was not affected by pre-treatment of nuclear extract with RNase, indicating that DEK associates with one or more SR proteins through protein-protein interactions and is not tethered to these proteins by RNA. Given that the association between DEK and SRm160/300 represents a potentially novel link between oncogenesis and pre-mRNA splicing, we next determined whether or not DEK is associated with splicing complexes.

DEK associates with splicing complexes assembled in vitro

Remarkably, rAb-DEK specifically and efficiently immunoprecipitated splicing complexes assembled on a constitutively spliced pre-mRNA substrate PIP85A (Figure 7). Similar to rAb-SRm300, which was assayed in parallel as a positive control for splicing complex immunoprecipitation, rAb-DEK preferentially immunoprecipitates splicing complexes containing exon sequences over the

complex containing the excised intron-lariat product. Control antisera, including pre-immune serum and a rabbit anti-mouse Ig antibody, did not immunoprecipitate splicing complexes. These findings are consistent with the evidence presented in Figure 6 that DEK associates with SRm160/300, which was previously demonstrated to preferentially associate with exon-containing splicing complexes.

The results indicate that DEK, a protein that has previously been implicated in myeloid leukemias, is associated with one or more SR proteins in splicing complexes. Future studies as part of the Idea Award research will extend these observations and address the functional significance of this new interaction.

Discussion (Part II)

The results from Year 1 of the Idea award research have identified an association between the putative oncoprotein DEK and the splicing process. DEK, together with several SR proteins, associates with the SRm160/300 splicing coactivator as well as with pre-mRNA splicing complexes *in vitro*. In addition, we have found recently that it is enriched in nuclear speckle structures containing splicing factors *in vivo* (data not shown). These results are consistent with a possible function of DEK in pre-mRNA splicing. Among the possibilities are that it is involved in modulating the activity of one or more SR-related proteins in general and/or regulated splicing. The identification of DEK in association with splicing components represents one of the first examples of a physical link between a factor involved in oncogenesis and pre-mRNA splicing. Future investigations will address the functional significance of this interaction.

Previously, DEK was identified as a fusion partner of the nucleoporin CAN arising from a (6;9) chromosomal translocation in a subclass of patients with acute myeloid leukemia (AML). In these patients, the N-terminal two thirds of DEK is fused in-frame to the C-terminal two-thirds of CAN, as a result of a translocation at intronic sequences within the DEK and CAN genes. The finding of another subclass of myeloid leukemias involving the identical portion of CAN fused to another protein, SET, suggested that the oncogenic potential of the DEK/CAN and SET/CAN fusion proteins may be due to disruption of the normal

functions of CAN. CAN is normally associated with nuclear pore complexes, with the majority of the protein located on the cytoplasmic face of the nuclear complex. In contrast, both the DEK/CAN and SET/CAN fusion proteins are predominantly nuclear. As has been speculated previously, it is possible that the redistribution of CAN by either DEK or SET is responsible, at least in part, for the oncogenic potential of the fusion proteins. However, it is also possible that an alteration to the normal function of DEK contributes to oncogenesis, which would be consistent with the observation that the disease profiles of patients with DEK/CAN and SET/CAN fusions are distinct. Previous work has identified DEK as a DNA binding protein that binds to a T/G-rich sequence defined as the “pets” (peri-ets) site ” site, an element that is adjacent to two binding sites of the ets family member Elf-1 in the HIV-2 enhancer. Enhancer activation by a number of signaling processes is mediated through the ets site and it was speculated that DEK functions in mediating transcriptional activation through the enhancer. Together with the data in the present study, it possible that DEK has functions in both transcription and pre-mRNA processing.

Interestingly, DEK has also been reported to have “anti-oncogenic” effects since it partially suppresses the transformation-prone phenotype of cells in patients with ataxia-telangiectasia (A-T). This is of particular interest since A-T patients are susceptible to developing breast and other hematological malignancies. Our observation that DEK associates with SR proteins and assembled splicing complexes raises the intriguing possibility that diseases including myeloid

leukemias in which DEK is altered, may arise in part as a consequence of a loss of the normal function of DEK in pre-mRNA processing. Future investigations as part of the IDEA award research will focus on elucidating the functional significance of the interaction of DEK with pre-mRNA splicing components.

Relevance to the Statement of Work objectives

During the first year of the Idea Award research we have developed two complementary strategies for the identification and characterization of factors that function in pre-mRNA processing: (1) a splicing complex affinity selection assay that permits the detection of SR protein-splicing complex interactions, and (2), a fractionation strategy for isolating new SR and SR-associated proteins.

During an early phase of our work, we attempted to establish conditions for performing the isolation of factors that regulate CD44 pre-mRNA alternative splicing by the cell sorting approach described in the original grant proposal. However, our initial experiments did not result in the successful identification of appropriate cell types that have robust and reproducible differential CD44 exon inclusion/exclusion signals that could be exploited for such a screen. Although further work is needed in this direction, our success in using the biochemical purification strategy described above to identify DEK and other candidate SR-associated proteins that may function in pre-mRNA processing has in essence achieved one of the goals set out in the original SoW. The connections between DEK, myeloid leukemia and A-T (see Discussion section) provides an opportunity to elucidate new interactions in pre-mRNA processing and how these interactions may be altered in cancers. This work is therefore highly relevant to the original proposal and will form the focus of year 2 of the Idea Award research.

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Idea Award Publications (1998-1999)

1. Eldridge, A.E., Li, Y., Sharp, P.A. and Blencowe, B.J. (1999). The SRm160/300 splicing coactivator is required for exon-enhancer function. *Proc. Natl. Acad. Sci. USA.* 96: 6125-6130.
2. Blencowe, B.J., Bowman, J.A.L., McCracken, S. and Rosonina, E. (1999). SR-related proteins and the processing of mRNA precursors. *Biochem. and Cell Biol.* 77: 1-15.
3. Li, Y. and Blencowe, B.J. (1999). Distinct factor requirements for exonic splicing enhancer function and binding of the U2 snRNP auxiliary factor to the polypyrimidine tract. *J. Biol. Chem.* In Press.
4. Blencowe, B.J. (1999). Exonic splicing enhancers: mechanism of action, diversity and role in human genetic diseases. *TiBS.* In press.

Figure Legends (PART 1)

Figure 1.

ESE-dependent association of SR proteins and hTra2 β with the dsx pre-mRNA.

A. In vitro splicing of biotinylated dsx pre-mRNAs. Radiolabeled and biotinylated dsx pre-mRNAs without an ESE (lane 1) or with an ESE containing three (lane 2) or six (lane 3) GAA repeats in the 3' exon were incubated in splicing reactions for 40 min. RNA recovered from the splicing reactions was analyzed on a 15% denaturing polyacrylamide-urea gel. The RNA intermediates and products of the splicing reaction are indicated.

B. Biotinylated dsx pre-mRNAs as described in (A) were transcribed cold and incubated in splicing reactions for 15 min prior to selection on streptavidin agarose beads (see Experimental Procedures). Selections were performed from splicing reactions incubated without pre-mRNA (lane 3), with a non-biotinylated dsx pre-mRNA (dsx(GAA)₆-bio, lane 4), with a biotinylated dsx pre-mRNA lacking an ESE (dsx Δ E, lane 5), with a biotinylated dsx pre-mRNA containing a partial ESE of three GAA repeats (dsx(GAA)₃+bio, lane 6), or with a biotinylated dsx pre-mRNA containing an ESE of six GAA repeats (dsx(GAA)₆+bio, lane 7).

Proteins recovered from the affinity selected splicing complexes were separated on a 12% SDS polyacrylamide gel and analyzed by immunoblotting with the monoclonal antibody mAb104, which recognizes a phosphoepitope shared between many RS domain proteins. Total nuclear extract, corresponding to approximately ~4% of the amount of extract used in each affinity selection, was separated as a marker for 104-reactive SR proteins in lane 1. Sizes markers and the sizes of defined SR and hTra2 proteins are indicated (in kDa).

C. Splicing complexes affinity selected in (B) were separated on a 12% SDS-polyacrylamide gel and immunoblotted with a polyclonal antibody specific for hTra2 β . Samples loaded in lanes 1-5 correspond to those in lanes 1,4-7 in (B).

Figure 2.

U2AF-65kDa binds to the dsx pre-mRNA equally in the presence and absence of the ESE.

A. Splicing complexes affinity selected as in Fig. 1B were analyzed by immunoblotting with a polyclonal antibody specific for U2AF-65kDa (30). The samples loaded in lanes 1-5 correspond to those in Fig. 1C.

B. Complexes selected from splicing reactions containing a biotinylated dsx(GAA)₆ pre-mRNA (lane 2) or a control biotinylated RNA consisting of a 5'-

half pre-mRNA substrate lacking a polypyrimidine tract (lane 3, see Experimental Procedures). The samples were separated on a 12% SDS polyacrylamide gel and immunoblotted with the anti-U2AF-65kDa antibody. The amount of nuclear extract loaded in lane 1, and the amount used in each affinity-selection in lanes 2 and 3, is as described in Fig. 1B.

Figure 3

Time course of association of SR proteins and U2AF-65kDa with the dsx pre-mRNA.

Biotinylated dsx(GAA)₆ pre-mRNA was incubated in a splicing reaction for the different time periods indicated, affinity selected on streptavidin agarose beads, and the bound complexes were analyzed by immunoblotting with mAb104 (A) and with the anti-U2AF-65kDa antibody (C). The amount of nuclear extract loaded in lane 1 and the amount used in each affinity selection is as described in Fig. 1B. Sizes of the defined SR proteins and hTra2 β recognized by mAb104 are indicated in (A).

(B) Supernatant fractions from the affinity selections in (A, C) were separated on a 12% SDS polyacrylamide gel and analyzed by western blotting with mAb104.

Figure 4

Binding of U2AF-65kDa to the dsx(GAA)₆ pre-mRNA requires U1 snRNP.

Splicing complexes assembled on the biotinylated dsx(GAA)₆ pre-mRNA were affinity-selected from splicing reactions incubated for 10 min containing either “mock”-depleted nuclear extract (lane 2), U1 snRNP-depleted nuclear extract (lane 4), U2 snRNP-depleted nuclear extract (lane 5), or an equal mixture of the U1 and U2 snRNP-depleted nuclear extracts (lane 6). A control selection was performed from a reaction containing “mock”-depleted extract and non biotinylated dsx(GAA)₆ pre-mRNA. Proteins recovered from the affinity-selected complexes were separated on a 12% SDS polyacrylamide gel and analyzed by immunoblotting with the anti-U2AF-65kDa antibody. The amount of “Total” extract separated in lane 1, and the amount used for each affinity selection, is as described in Fig. 1B.

Figure Legends (PART 2)

Figure 5. DEK co-purifies with SR proteins

- A. Fractionation scheme for the mAb-104 antigens.
- B. Coomassie-stained SDS polyacrylamide gel of the Q-Seph-0.3 fraction.
The identities of the abundant polypeptides, migrating at 130kDa, 50kDa, 34kDa and 20kDa, were identified by MALDI-TOF mass spectroscopy as Hel117, DEK, SC35, and eIF1A, respectively.
- C. Mab-104 immunoblot of the Q-Seph-0.3 fraction obtained by the scheme in (A). Size markers are indicated in kDa. SR proteins of ~75kDa, 50kDa and 30kDa are detected in the fraction.

Figure 6. DEK associates with SR proteins

Immunoprecipitates were collected with mAb-B1C8 (lanes 5,8), mAb-B3 (lanes 4,7) and a control antiserum (rab-anti-mouse; lanes 3,6) from HeLa nuclear extract pre-incubated with (lanes 6-8) or without RNase (lanes 3-5) using, and were immunoblotted with rAb-DEK. The corresponding total HeLa nuclear extracts treated without or with RNase, corresponding to ~5% of the amount of nuclear extract used in each immunoprecipitation is shown in lanes 1 and 2.

Figure 7. Association of DEK with splicing complexes

Immunoprecipitation of splicing complexes with rAb-DEK from reactions incubated for 1 hr containing PIP85A pre-mRNA. RNA recovered following immunoprecipitation (lanes 2-4) and RNA recovered directly from a parallel splicing reaction (lane 1), was loaded on a 15% denaturing polyacrylamide urea gel. RNA loaded in lane 1 represents 25% of the total amount recovered, whereas RNA loaded in lanes 2-4 from each immunoprecipitation represents 50% of the total amounts recovered. Immunoprecipitations were performed with a pre-immune serum (lane 2), rAb-DEK (lane 3), and a positive control antibody (rAb-SRM300; lane 4).

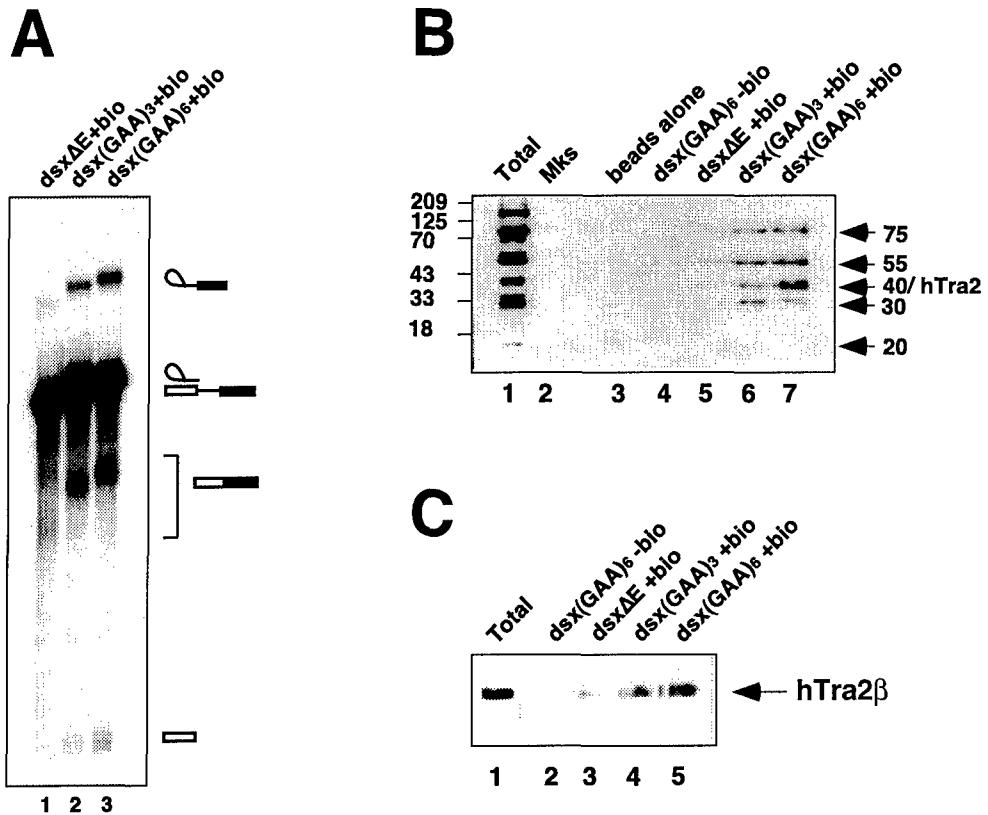
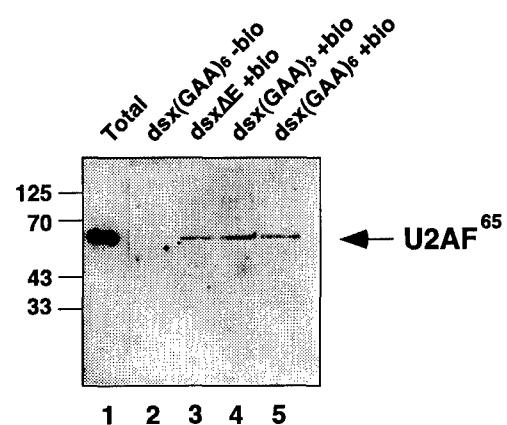


Figure 1
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A



B

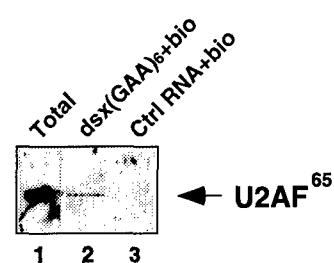


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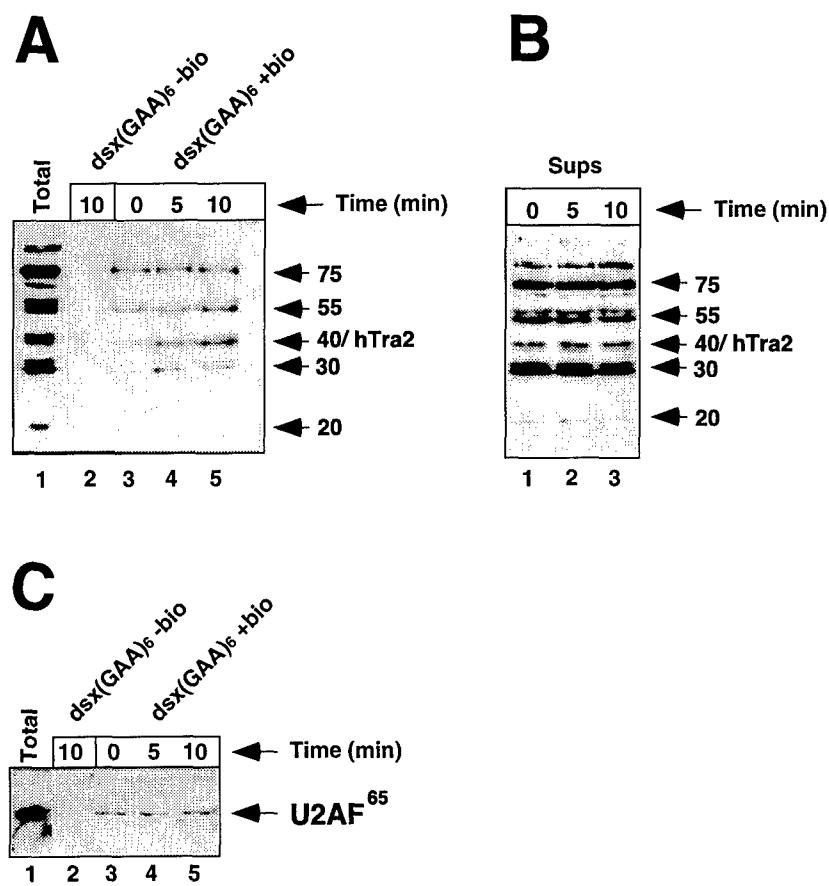


Figure 3
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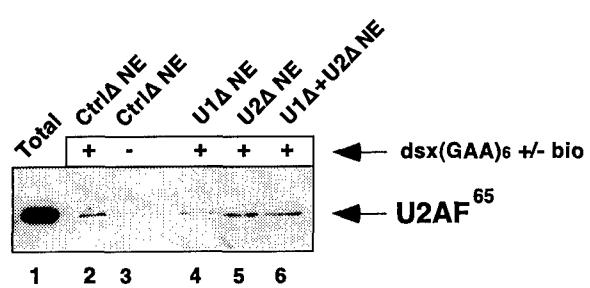


Figure 4
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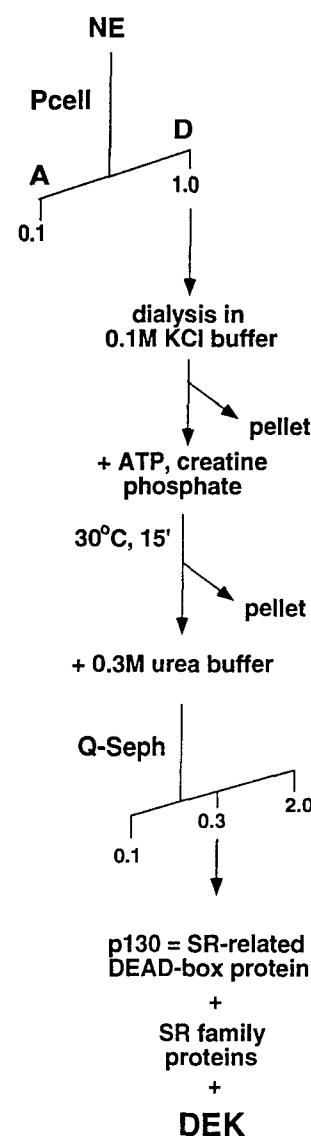


Figure 5A

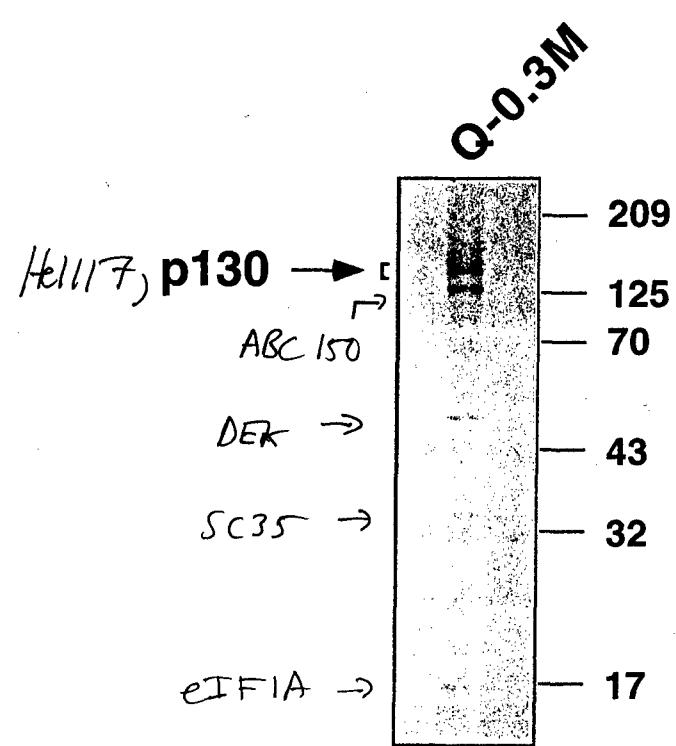


Figure 5B

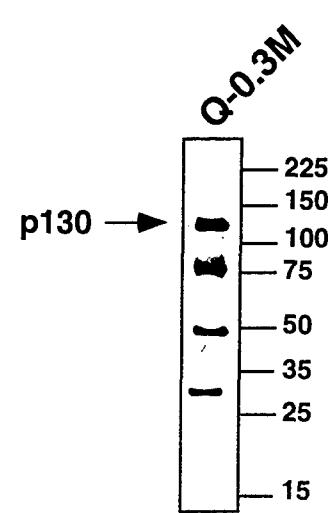


Figure 5C

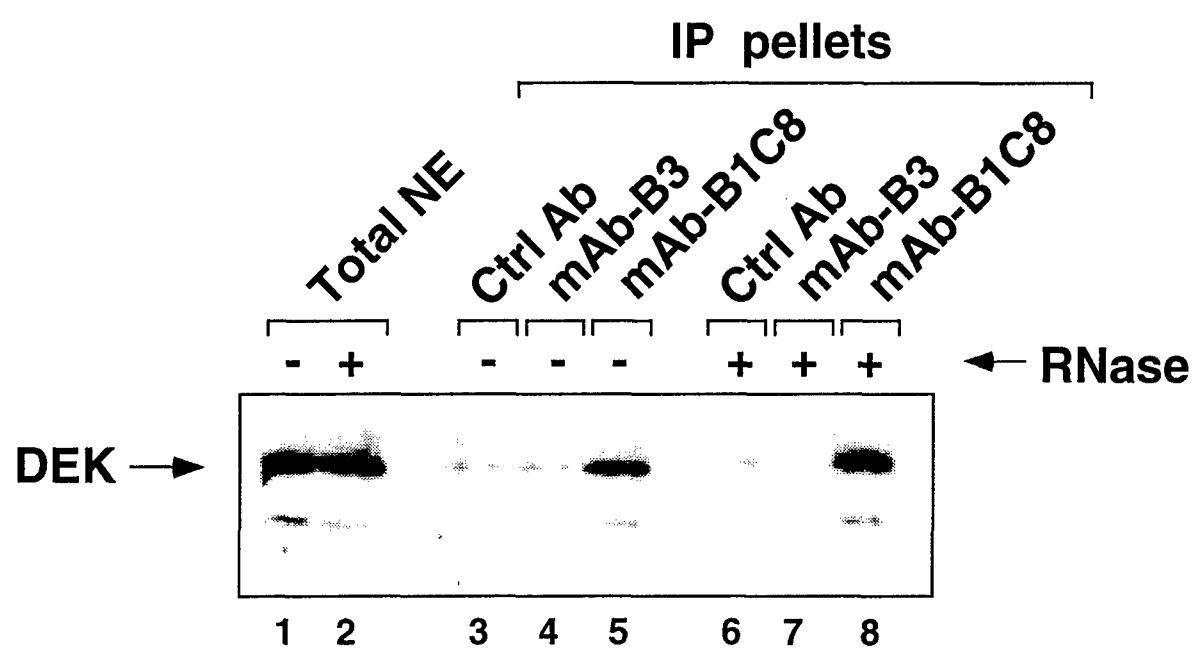


Figure 6

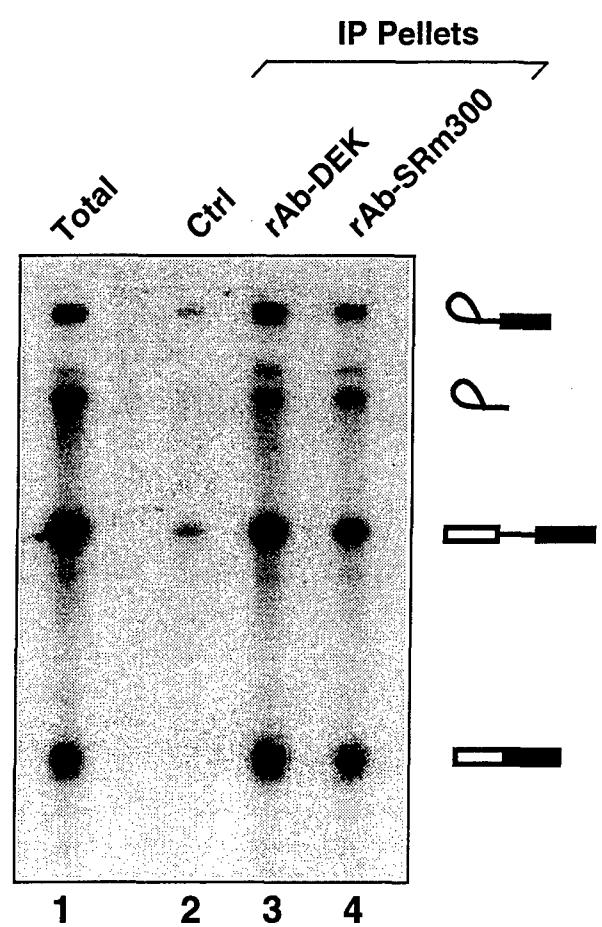


Figure 7



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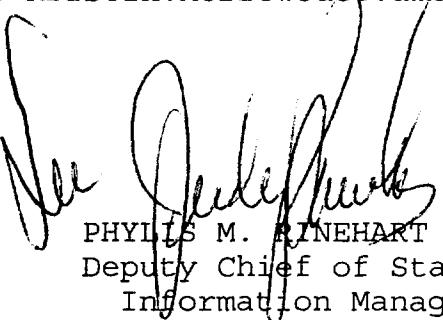
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